

**Low-LET Bystander Effects In Cells *In Vitro* Are Significantly  
Less Than Published For High-LET Radiation**

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There have been several approaches to quantitatively measure low dose bystander effects from low-linear energy transfer (LET) radiation sources, including studies involving media transfer from irradiated cells to unirradiated cells, mixing irradiated cells with non-irradiated cells, and co-culturing of cells labeled with tritiated thymidine with non-labeled cells. Few studies however have been able to analyze the spatial range of effectiveness of bystander effects from directly targeted cells with sufficient measured precision of the dose profiles, and adequate statistics. We are addressing these issues in bystander studies with human mammary epithelial cells (HMEC) irradiated with a synchrotron-based 12.5 keV X-ray Microbeam line 10.3.1 at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory. The characteristics of the fan beam can be easily changed to deliver a dose stripe of variable width from 4 to 200  $\mu\text{M}$  with a flat-top dose distribution.

Cultures of HMEC that are phenotypically normal, non-malignant, but with an extended life span in culture are grown in Lab-Tek 4-well chamber plastic slides and irradiated in studies presented here with precise stripes of dose 18mm long and up to 100 microns wide, ranging from 10 cGy to 100 cGy. The 100 microns wide 12.5 keV beam is produced with a multilayer monochromator and a precision slit system. The intensity of the beam is calibrated before sample irradiation with both an argon-filled ion chamber and a calibrated silicon diode. Each well on the slide measures 10mm x 22mm. The slide is covered with a liquid-tight, but gas permeable membrane to permit it being held vertical during the 2-3 minute irradiation of the slide with the fixed horizontal beam. A single X-ray dose stripe is delivered in three of the four wells to allow for an unirradiated control well by scanning the sample at a fixed rate across the beam. In some experiments two dose stripes have been delivered per well with variable distances between them to determine if bystander effects can be additive between two targeted sources.

Samples are processed for the expression of the radiation-induced protein marker TP53<sup>serine 15</sup> phosphorylation with fluorescent immunohistochemistry in a time course from 10 minutes to several hours after exposure with variable times of fixation by  $-20^{\circ}\text{C}$  100% methanol. Using fluorescent microscopy on a high-precision-controlled microscope stage and fiducial marked references, the physical locations of the dose stripes are mapped exactly to the location of the biological responses. Montage images (5 X 6 frames) are acquired that cover an area of 770  $\mu\text{m}$  X 950  $\mu\text{m}$ . Up to 6 montage images are acquired along each dose stripe. Each montage image contains several 1000 cells. Analysis of radiation-induced fluorescent signals was completed with a computer-based system that objectively measures the signal on individual DAPI-stained cell nuclei in both the targeted dose stripe and untargeted adjacent cells. In effect, we are able to objectively evaluate the widening of the dose stripe with time to include bystander cells

using five or more montages of 30 images representing several thousands cells for each experimental sample. Data from replicate experiments are combined. Some samples were pretreated with 40  $\mu$ M lindane for 1 hr to inhibit gap junction intracellular communication (GJIC).

We have made a quantitative physical assessment of our 12.5 keV X-ray Microbeam beam profile, and measurements of dose- and time-dependent bystander effects on TP53<sup>ser15</sup> phosphorylation in the untargeted regions adjacent to the dose stripe. Using a 100 cGy dose we have analyzed the response profile from 6 min to 60 minutes in steps of 2-3 minutes. We are currently analyzing similar data 6-30 min after a 20 cGy dose. Data analyses are also in progress on the effects of lindane and the double dose stripes separated by varying distances. In general we find that there is a time- and dose-dependent response of the integrated fluorescence response. However, we find that the spatial distance of Bystander Effects for our low-LET radiation is less than 50  $\mu$ m. This is significantly different than the effect published for high-LET experiments.

*This work was supported by the U.S. DOE's Low Dose Radiation Research Program under Contract No. DE-AC02-05CH11231. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.*